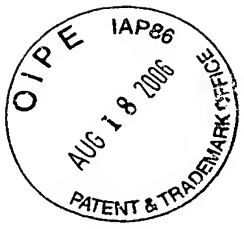
APPENDIX D



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Detection of *APC* and *k-ras* Mutations in the Serum of Patients with Colorectal Cancer

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ABSTRACT: Detection of tumor DNA in peripheral blood of patients with colorectal cancer (CRC) may allow early diagnosis of tumor disease and be of prognostic value. However, a reliable tumor marker detectable in the serum of patients with this disease is missing. Because *k-ras* and *APC* mutations occur frequently and at an early stage in CRCs, these mutations might also be detected in the serum of CRC patients and serve as tumor markers. Hence, tumor tissues of CRC patients were examined for the presence of mutations in the *k-ras* and *APC* genes. If a mutation was detected in the tumor, the serum of the patient was screened subsequently for the presence of this mutation. *K-ras* mutations were detected in 22 of 30 colorectal tumor tissues, but only in six patients was the mutation identified in their serum samples. Mutations of the *APC* gene were identified in 25 of 65 tumors; 20 of these 25 patients showed the respective mutation in their serum. Given their higher detection rate, *APC* mutations could be a more informative serum marker than *k-ras* in CRC patients.

KEY WORDS: *APC* mutations, colorectal cancer, *k-ras* mutations, serum marker.

The presence of tumor DNA in the plasma of cancer patients was observed more than two decades ago.[1] The presence of extractable amounts of DNA in the plasma of patients with a variety of malignancies and its absence in healthy controls could be demonstrated.[2] Furthermore, higher amounts of serum DNA in patients with metastatic tumor disease than in patients with localized disease and decreasing amounts of DNA after tumor regression have been shown.[1] However, it is only since publication of the milestone papers on the detection of tumor DNA in the plasma of patients with small-cell lung cancers and head and neck cancers[3,4] that an ever-increasing number of articles on the detection of tumor DNA in the serum or plasma of cancer patients have been published.[5–8] This approach may lead to improvement in the diagnosis of cancers in several ways: (1) The detection of circulating tumor DNA may be a marker of metastasis and lead to a more precise staging of tumor disease, thus serving as a prognostic marker; (2) searching for tumor DNA in serum may be useful for detecting recurrent disease after surgery or for planning chemotherapy; and (3) circulating tumor DNA may prove to be a useful tumor marker in cancer screening.

In studies on the detection of tumor DNA in the serum of colorectal cancer (CRC) patients published thus far, microsatellite instability (MSI), loss of heterozygosity, *k-ras* mutations, and *p53* mutations were chosen as diagnostic parameters. MSI has been shown to be a valuable serum marker in patients with small-cell lung cancer and head and neck cancers.[3,4] However, MSI has been shown to be present in only 10% to 15% of CRC patients.[9] Furthermore, in none of 15 tumors showing MSI and in none of 31 tumors with loss of heterozygosity could Hibi et al.[8] trace these microsatellite variations to the sera of their patients.

K-ras mutations are common in CRC, and approximately 80% of the mutations are found in codon 12 of the gene.[10,11] Data regarding *k-ras* mutations in the serum of CRC patients is contradictory. Though some investigators were able to detect mutant *k-ras* in the serum of 86% of CRC patients whose tumors harbored a *k-ras* mutation,[5,6] Hibi et al.[8] found a mutation in the serum in only 3 of 16 cases. In contrast, the latter authors were able to detect *p53* mutations in the serum of 7 of 10 patients whose tumors had a *p53* mutation. However, a *p53* mutation was detected in only 10 of a total of 33 CRC patients. The authors conclude that

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the use of *p53* is limited by the large number of different mutations within the gene and that other genetic targets such as *APC* might be a useful marker to study.

It is well-known that somatic *APC* mutations occur as first events in the cascade of molecular alterations within the adenoma-carcinoma sequence.[12] Germline mutations in the *APC* gene are responsible for familial adenomatous polyposis, an autosomal dominant precancerous condition characterized by the appearance of hundreds to thousands of colorectal adenomas. *APC* is a relatively large gene (>8 kb), and germline mutations in familial adenomatous polyposis patients are widely spread over major parts of its coding sequence. Most somatic mutations detected in sporadic CRC, however, are limited to the so-called mutation cluster region (MCR) in exon 15, encompassing codons 1286 through 1513.[13] Mutations occurring in this relatively small part of the gene can easily be detected by heteroduplex or single-strand conformation analysis.

In this study, we set out to compare the potential of *k-ras* mutations and *APC* mutations as serum markers in patients with CRC.

MATERIALS AND METHODS

Patients

We included in the study 65 patients with CRC after obtaining their written informed consent. All tumors were histologically characterized as adenocarcinomas and were classified according to Union Internationale Contre le Cancer (UICC) stages: 23 patients had UICC stage I tumors, 7 stage II, 31 stage III, and 4 stage IV tumors, respectively. Peripheral blood samples were obtained 1 day before and at two intervals (1 and 10 days) after surgical resection of the tumor. Serum was prepared immediately and stored at –70°C.

DNA Extraction

Tumor DNA of the CRC patients was extracted from paraffin-embedded microdissected tumor tissue using the QiaAmp Tissue Kit (Qiagen, Hilden, Germany). DNA from serum samples was extracted by the QiaAmp Blood & Body Fluid Protocol (Qiagen).

Detection of *k-ras* Codon 12 Mutations

Tumor DNA was amplified by polymerase chain reaction (PCR) using the primers and PCR conditions as described by Jiang et al.[14] and Enrius et al.[15] The forward primer (primer A: 5′-ACT GAA TAT AAA CTT GTG GTA GTT GGA CCT-3′) contains a mismatched nucleotide at the 3′ end that generates a restriction site for BstNI in wild-type DNA but not in the DNA that harbors a mutation at codon 12. DNA of the colorectal tumor cell line SW480 was used as a positive control for the *k-ras* codon 12 mutation. PCR products were digested with BstNI and separated on polyacrylamide gels followed by silver staining.

Detection of *APC* Mutations

Somatic mutations in the MCR of the *APC* gene (codons 1286–1513) in the tumors were detected by heteroduplex and single-strand conformation analysis followed by silver staining, as described previously (Figure 1).[16] If a mutation was detected, the existence of this mutation was excluded in the control DNA

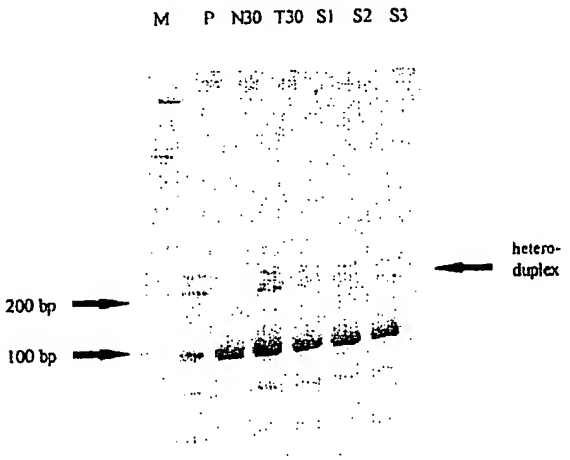

FIGURE 1. Detection of the 5 bp deletion at codon 1309 of the *APC* gene in tumor tissue (T30) and in serum samples of patient 30 taken 1 day before (S1), 1 day after (S2), and 10 days after operation (S3). The heteroduplex bands are not present in DNA from lymphocytes of the patient (N30). (P = patient with familial adenomatous polyposis, with a germline mutation at codon 1309, as a positive control for the mutation; M = molecular weight marker with 100 bp ladder.)

derived from the leukocytes of the same patient to rule out a germline *APC* mutation.

Detection of Tumor DNA in Serum Samples

In the patients whose tumors exhibited *k-ras* or *APC* mutations, we screened their serum samples for the presence of these mutations. Essentially, the same detection methods were applied in the sera as in the tumors for *k-ras* or *APC* mutations. However, owing to the well-known fact that the tumor DNA present in the serum of cancer patients is partially degraded,[17] the MCR of the *APC* gene was examined in seven overlapping fragments with a length of approximately 150 bp each. (Primers and PCR conditions can be obtained on request.) Mutations detected by this method were subsequently confirmed by sequencing of the serum DNA on an ABI 377 sequencer (Perkin Elmer, Weiterstadt, Germany) (Figure 2).

In cases in which the mutations could not be traced to the sera, a reamplification was performed as described by Deuter and Müller.[18] Briefly, the portion of the polyacrylamide gel corresponding to the presence of aberrant bands in the respective tumors was excised and incubated with water at 60°C for 1.5 hours; 5 µl was reamplified, using the same primers and PCR conditions as outlined for *APC* mutations and primer set A and D (primer D: 5′-TCA TGA AAA TGG TCA GAG AAA CC-3′) for *k-ras* mutations.

RESULTS

We examined CRC tissue from 65 patients for *APC* and *k-ras* mutations that might be detectable in the patients' sera. The presence of tumor-specific mutations was followed up in the three serum samples obtained from each patient, 1 day before and 1 and 10 days after tumor excision.

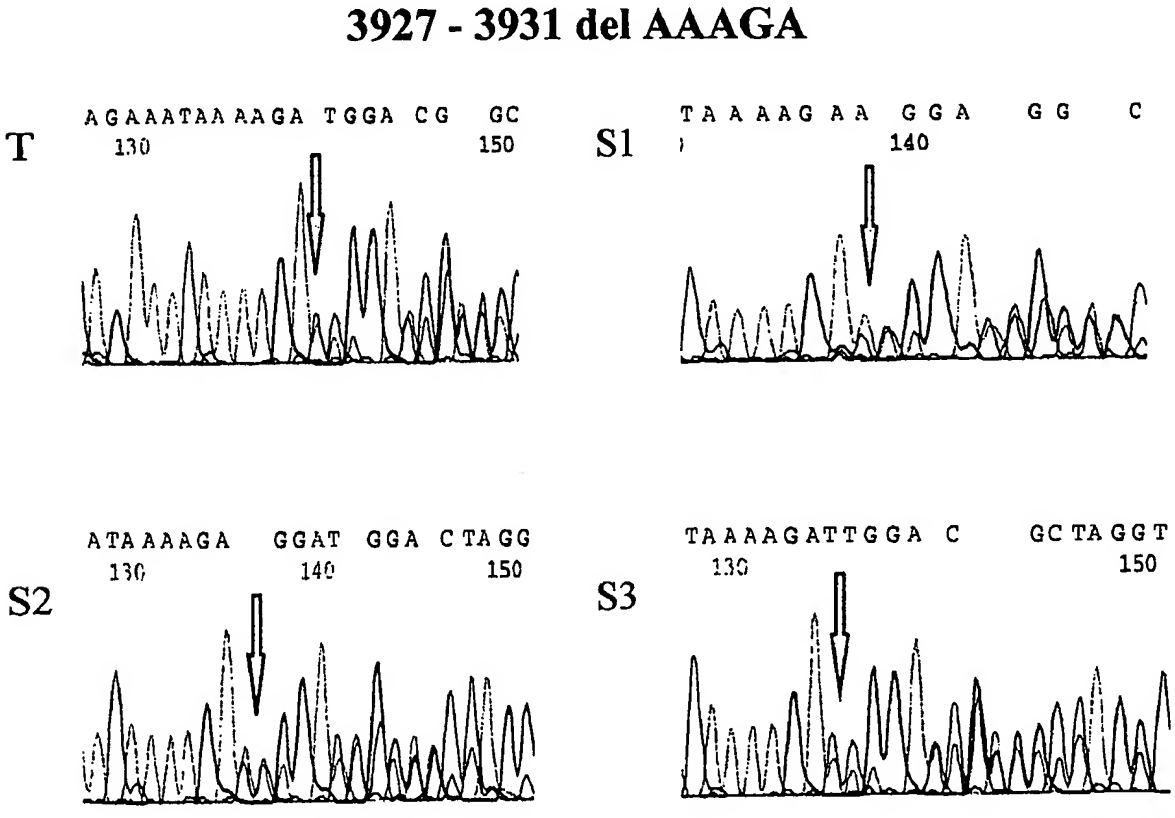

FIGURE 2. Sequencing of the DNA derived from tumor tissue (T) and serum samples of patient 30 taken 1 day before (S1), 1 day after (S2), and 10 days after operation (S3).

Detection of *k-ras* Mutations

In a first step, 30 CRC patients were examined. By using the mutant allele–specific restriction method,[14,15] we were able to detect mutations in codon 12 of the *k-ras* gene in 22 of the 30 tumors (73%; Table 1). However, when the same method was applied to the serum of the patients, the *k-ras* mutation was detectable in only one patient. To increase the detection rate in the serum samples, the position characteristic of the mutant allele was excised from the gel, eluted, and reamplified.[18] After reamplification, the mutation was detectable in the serum samples of another five patients. Thus, we ended with a detection rate of 27% (6 of 22 patients). In all six patients in whom the *k-ras* mutation could be traced to the serum, all three serum samples showed the mutation (see Table 1).

Detection of Mutations in the *APC* Gene

We also screened the tumors for mutations in the MCR of the APC gene. In 13 of the 30 patients (43%), a mutation was detected in the tumor. In 11 of these 13 patients, the mutations could be traced to the serum (see Figure 1). In one serum sample, no PCR product could be obtained. In six cases, the aberrant bands observed by heteroduplex analysis of the serum samples were excised from the gel and sequenced, to prove that they are due to the same mutations as those detected in the tumors (see Figure 2).

From this series of experiments, we determined that *k-ras* mutations were present in the majority of CRC cases (22 of 30; 73%) but could be detected in only a minority of the serum samples (6 of 22; 27%). On the other hand, somatic *APC* mutations were de-

TABLE 1
Detection of *k-ras* Mutations in Colorectal Tumors and Serum Samples of 22 of 30 Patients

Patient	UICC stage	Tumor	Serum		
			S1	S2	S3
T7	I	+	–	–	–
T20	I	+	+(GE)	+(GE)	+(GE)
T23	I	+	–	–	–
T26	I	+	–	–	–
T28	I	+	–	–	–
T4	II	+	+	+	+
T6	II	+	–	–	–
T9	II	+	–	–	–
T11	II	+	+(GE)	+(GE)	+(GE)
T27	II	+	–	–	–
T8	III	+	–	–	–
T15	III	+	+(GE)	+(GE)	+(GE)
T16	III	+	NPP	NPP	NPP
T21	III	+	–	–	–
T22	III	+	–	–	–
T24	III	+	+(GE)	+(GE)	+(GE)
T29	III	+	+(GE)	+(GE)	+(GE)
T30	III	+	–	–	–
T31	III	+	–	–	–
T32	III	+	NPP	NPP	NPP
T3	IV	+	–	–	–
T18	IV	+	–	–	–
		Σ22	Σ6	Σ6	Σ6

UICC = Union Internationale Contre le Cancer; S1 = serum 1 day before operation; S2 = serum 1 day after operation; S3 = serum 10 days after operation; + indicates mutation detected; – indicates no mutation detected; (GE) = detectable after extraction from gel and reamplification; NPP = no polymerase chain reaction product.
Note: No *k-ras* codon 12 mutation was identified in eight tumors.

tected in only 43% of all tumors (13 of 30) but could be traced to most of the corresponding serum samples (11 of 13; 85%). Thus, *APC* mutations were more frequently detectable in the serum of our CRC patients, suggesting that mutant *APC* might be a more sensitive tumor marker as compared to *k-ras* codon 12 mutations.

Therefore, in a second series of 35 CRC patients, we examined tumor and serum samples for APC mutations only. In 12 tumors, an *APC* mutation was detected that could be traced to the corresponding serum samples in 9 cases. Thus, the results were similar to those obtained in the first 30 patients (Table 2).

In our study, we observed a tendency toward a stage-dependent difference in the occurrence of *APC* mutations in the serum of CRC patients, which may point to its possible significance as a prognostic marker. The percentage of *APC* mutations detected in the tumors did not differ among UICC stage I, II, III, and IV tumors: Hence, the well-known early occurrence of mutations in the *APC* gene has been confirmed. Overall, *APC* mutations have been detected in 25 of the 65 tumors examined (see Table 2). Subsequently, the serum samples of these 25 patients were screened for the presence of the respective mutations. In three patients whose *APC* mutations could not be detected in their serum samples, no PCR products were obtained at any of the three blood sampling events,

TABLE 2
Detection of *APC* Mutations in Colorectal Tumors and Serum Samples of 25 Patients

Patient	UICC stage	Tumor	Serum			Sequencing of serum DNA
			S1	S2	S3	
T5	I	+	+	+	+	ND
T25	I	+	+	+	−	ND
T26	I	+	+	+	+	nt4372 del C
T28	I	+	+	+	+	nt4087-4088 del AA
T34	I	+	NPP	NPP	NPP	
T36	I	+	−	−	−	
T55	I	+	+	+	−	ND
T4	II	+	+	+	−	ND
T9	II	+	−	−	−	ND
T27	II	+	+	+	+	nt4087-4088 del AA
T53	II	+	+	+	+	ND
T14	III	+	+	+	+	nt4463 del T
T16	III	+	NPP	NPP	NPP	
T21	III	+	+	+	+	ND
T30	III	+	+	+	+	nt3927-3931 del AAAGA
T31	III	+	+	+	+	ND
T37	III	+	+	+	+	ND
T39	III	+	NPP	NPP	NPP	
T42	III	+	+	+	+	ND
T46	III	+	+	+	+	nt3927-3931 del AAAGA
T51	III	+	+	+	+	ND
T56	III	+	+	+	+	ND
T18	IV	+	+	+	+	nt3927-3931 del AAAGA
T52	IV	+	+	+	+	ND
T60	IV	+	+	+	+	ND
		Σ25	Σ20	Σ20	Σ16	Σ7

UICC = Union Internationale Contre le Cancer; S1 = serum 1 day before operation, S2 = serum 1 day after operation, S3 = serum 10 days after operation; + indicates mutation detected; − indicates no mutation detected; ND = not done; NPP = no polymerase chain reaction product.
Note: In 40 tumors, no mutation in the mutation cluster region of the *APC* gene could be detected. Of these tumors, 10 were UICC stage I, 8 UICC stage II, 14 UICC stage III, and 8 UICC stage IV.

possibly owing to the presence of inhibitors in the patients' serum. Of the remaining 22 patients whose tumors showed *APC* mutations, 10 had UICC stage I and II tumors and 12 had UICC stage III and IV tumors. Whereas in all 12 patients with stage III and IV tumors the respective *APC* mutation could be traced to their serum samples, the mutations could be detected in only 8 of the 10 patients with stage I and II tumors. Furthermore, in four of these eight patients, the respective mutation was no longer detectable in the serum sample taken at day 10 postoperatively. In contrast, none of the 12 patients at stages III and IV had lost the *APC* mutations from their serum taken at day 10 postoperatively (see Table 2). However, any comment on the significance of this difference must be prefaced by information on follow-up of these patients.

DISCUSSION

According to the literature, 20% to 50% of all CRCs harbor somatic mutations in the *k-ras* gene, and 80% of these occur in codon 12 of the gene. By the mutant allele–specific restriction method we used, we were able to detect mutations in codon 12 of the *k-ras* gene in 22 of 30 tumors (73%; see Table 1). However, the mutation could be traced to the serum of only 6 of these 22 patients (27%). This finding is in agreement with the data published by Hibi et al.,[8] who found *k-ras* mutations in the serum in 3 of 16 CRC patients (19%) whose tumors exhibited *k-ras* mutations. In contrast, studies by Anker et al.,[5] de Kok et al.,[6] and Kopreski et al.[7] found a much higher proportion of mutant *k-ras* in the serum of CRC patients. Kopreski's group[7] reported on the detection of *k-ras* codon 12 mutations in the serum of 12 of 31 patients (39%), all of whom had metastatic disease.

Likewise, de Kok et al.[6] included in their study only patients with advanced disease (UICC stage IV tumors). They found *k-ras* codon 12 or 13 mutations in the serum of six of seven CRC patients (86%) whose tumors proved positive for a mutation. Anker et al.[5] also found mutations in the serum of six of seven patients whose tumors harbored *k-ras* mutations. In a subset of their patients, Anker's group[5] proved the presence of *k-ras* mutations in the serum through cloning and sequencing techniques to rule out the possibility of false-positive results.

False-positive results in the detection of *k-ras* mutations are due to the generation of PCR artifacts, which are likely to occur with increasing cycles of PCR. Therefore, the method of reamplification of the mutant allele we used could be prone to false-positive results, as a second round of 25 PCR cycles is used after the first PCR round consisting of 32 cycles. To rule out this possibility, the existence of the detected mutations should be confirmed by a second method (e.g., sequencing) in an independent PCR probe. However, this would make the diagnostic program even more laborious and expensive and not feasible in a routine setting. Furthermore, we could find *k-ras* mutations in the serum of only 27% of the patients, which confirms the results of Hibi et al.[8] Thus, we decided to complete the *k-ras* investigations after 30 patients and proceed with the *APC* study, as the results of the latter were more promising.

By screening colorectal tumors of 65 patients for somatic mutations in the MCR of the *APC* gene, we detected a mutation in 25 tumors (38.5%; see Table 2). This rate of mutations within the MCR agrees well with published data.[13,18,19] In 20 of the 25 cases, the respective mutations could be detected in the serum. In our hands, the screening for mutations within the MCR of the *APC* gene by nonradioactive single-strand conformation polymorphism and heteroduplex analysis is both rapid and simple, and the results obtained with mutated *APC* as a serum marker in CRC patients were more promising than results obtained with *k-ras*. Furthermore, we observed a tendency to a stage-dependent difference in the occurrence of *APC* mutations in the serum of CRC patients, which may point to a possible significance as a prognostic marker.

There were two considerations for taking the serum samples at three different time points: First, it is possible to detect tumor DNA in the serum postoperatively but not preoperatively, owing to the dissemination of tumor cells during surgical intervention. However, in all patients in whom we were able to detect mutant DNA in the serum, the mutant DNA was present both 1 day before and 1 day after tumor excision. We therefore conclude that this method is not useful for the demonstration of tumor cell dissemination by the surgeon.

Second, it is possible that mutations detected in the serum preoperatively are lost after surgery as an indication of complete tumor excision. Although we observed this, it happened in only a minority of cases and, even in four of ten patients at UICC stage I

and II tumors, the mutant DNA remained 10 days postoperatively. We therefore speculate that the last blood samples might have been taken too early, as more time may be needed for the mutant DNA to clear from the blood. However, the time point was chosen for practical reasons, because the patients usually stay in the clinic for approximately 10 days after surgery.

Taken together, despite the much higher proportion of *k-ras* mutations detected in tumors (73%) as compared to *APC* mutations (38.5%), in our hands *APC* is superior as a serum marker in patients with CRC, as we could trace the *k-ras* mutations to the serum of only 27% of the patients, whereas we could trace the *APC* mutations detected in the tumors to 80% of the corresponding serum samples. In conclusion, *APC* seems to be a good serum marker for the detection of tumor cell DNA from CRCs. Nonetheless, there remains a need to identify additional markers, as only 40% or so of all CRCs harbor mutations within the MCR of the *APC* gene. To estimate whether this serum marker is also a valuable indicator of prognosis in CRC patients, a longitudinal follow-up study of the patients is being conducted.

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